Attorney Docket No. 025455-113

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of	
Deborah Kim Glencross	Group Art Unit: 1651
Application No.: 10/754,711	Examiner: ALLISON M FORD
Filed: January 12, 2004	Confirmation No.: 1340
For: CELL ENUMERATION	

SUBMISSION OF CERTIFIED COPY OF PRIORITY DOCUMENT

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

The benefit of the filing date of the following priority foreign application in the foreign country of South Africa is hereby requested, and the right of priority provided in 35 U.S.C. § 119 is hereby claimed.

Country:	SOUTH AFRICA
Patent Application No.:	2001/5700
Filed:	July 11, 2001

In support of this claim, enclosed is a certified copy of said foreign application and which is identified in the original Oath/Declaration Acknowledgement of receipt of this certified document is requested.

Respectfully submitted,

BURNS, DOANE, SWECKER & MATHIS, L.L.P.

Date: May 16, 2005

By: _____
Alan E. Kopecki
Registration No. 25,813

P.O. Box 1404
Alexandria, Virginia 22313-1404
(703) 836-6620

711

Sertifikaat

REPUBLIEK VAN SUID AFRIKA

PATENT KANTOOR
DdepartemENT VAN HANDEL
EN NYWERHEID

Certificate

REPUBLIC OF SOUTH AFRICA

PATENT OFFICE
DEPARTMENT OF TRADE AND INDUSTRY

Hiermee word gesertifiseer dat
This is to certify that

the documents attached hereto are true copies of Forms P2, P6 provisional specification and drawings of South African Patent Application No. 2001/5700 in the name of National Health Laboratory Service

Filed : 11 July 2001

Entitled : Cell Enumeration

Geteken te **PRETORIA** in die Republiek van Suid-Afrika, hierdie 4th dag van **October 2004**

Signed at in the Republic of South Africa, this day of

.....
Registrar of Patents

FORM P.2

REPUBLIC OF SOUTH AFRICA — REGISTER OF PATENTS — PATENTS ACT, 1978

OFFICIAL APPLICATION NO.	LODGING DATE: PROVISIONAL	ACCEPTANCE DATE
22 01 20015700	22 11 JULY 2001	47
INTERNATIONAL CLASSIFICATION	**LODGING DATE: COMPLETE**	**GRANTED DATE**

FULL NAME(S) OF APPLICANT(S)/PATENTEE(S)

71 ~~ROUSSOS, Deborah Kim~~

AANSOEKERS VERVANG
APPLICANTS SUBSTITUTED

APPLICANTS SUBSTITUTED:	DATE REGISTERED
71 NATIONAL HEALTH LABORATORY SERVICE	12/7/2002
UNIVERSITY OF WITWATERSRAND	

ASSIGNEE(S)	DATE REGISTERED
71	

FULL NAME(S) OF INVENTOR(S)

72 ROUSSOS, Deborah Kim

PRIORITY CLAIMED	COUNTRY	NUMBER	DATE
N.B. Use International Abbreviation for country (See Schedule 4)	33 NIL	31 NIL	32 NIL

TITLE OF INVENTION

54 "CELL ENUMERATION"

ADDRESS OF APPLICANT(S)/PATENTEE(S)

Cnr DE KORTE AND HOSPITAL Streets
~~17, 5th Avenue, Houghton~~, Gauteng, Republic of South Africa
Boksburg Fontein JHG 2001

ADDRESS FOR SERVICE	A & A REF:
74 ~~ADAMS & ADAMS, Pretoria~~ SPOOR & FISHER SANDTON	V14729

PATENT OF ADDITION TO NO.	DATE OF ANY CHANGE
61	
FRESH APPLICATION BASED ON	**DATE OF ANY CHANGE**

A&A P202

REPUBLIC OF SOUTH AFRICA
PATENTS ACT, 1978
APPLICATION FOR A PATENT AND
ACKNOWLEDGEMENT OF RECEIPT
(Section 30(1) Regulation 22)

FORM P.1
(to be lodged in duplicate)

IC OF SOUTH AFRICA
REVENUE
11.7.01 R 060.00
INKOMSTE
REPUBLIEK VAN SUID AFRIKA
A&A REF HASR 4729 370

THE GRANT OF A PATENT IS HEREBY REQUESTED BY THE UNDERMENTIONED APPLICANT ON THE BASIS OF THE PRESENT APPLICATION FILED IN DUPLICATE

21	01	PATENT APPLICATION NO	2001700

71	FULL NAME(S) OF APPLICANT(S)

AANSOEKERS VERVANG
APPLICANTS SUBSTITUTED

~~ROUSSOS, Deborah Kim~~
NATIONAL HEALTH LABORATORY SERVICE
UNIVERSITY OF WITWATERSRAND

ADDRESS(ES) OF APPLICANT(S)

BEST AVAILABLE COPY

~~17, 5th Avenue, Houghton~~, Gauteng, Republic of South Africa
Cnr De Korte and
Hospital Streets Braamfontein Jhb 2001

54	TITLE OF INVENTION

"CELL ENUMERATION"

Only the items marked with an "X" in the blocks below are applicable.

[ ] THE APPLICANT CLAIMS PRIORITY AS SET OUT ON THE ACCOMPANYING FORM P.2. The earliest priority claimed is
Country: No: Date:

[ ] THE APPLICATION IS FOR A PATENT OF ADDITION TO PATENT APPLICATION NO 21 01

[ ] THIS APPLICATION IS A FRESH APPLICATION IN TERMS OF SECTION 37 AND BASED ON APPLICATION NO 21 01

THIS APPLICATION IS ACCOMPANIED BY:

X	A single copy of a provisional specification of 11 pages
X	Drawings of 3 sheets
	Publication particulars and abstract (Form P.8 in duplicate) (for complete only)
	A copy of Figure of the drawings (if any) for the abstract (for complete only)
	An assignment of invention
	Certified priority document(s). (State quantity)
	Translation of the priority document(s)
	An assignment of priority rights
	A copy of Form P.2 and the specification of RSA Patent Application No 21 01
X	Form P.2 in duplicate
X	A declaration and power of attorney on Form P.3
	Request for ante-dating on Form P.4
	Request for classification on Form P.9
	Request for delay of acceptance on Form P.4
	Extra copy of informal drawings (for complete only)

74	ADDRESS FOR SERVICE: ~~Adams & Adams, Pretoria~~

SPOOR & FISHER SANDTON.

Dated this 11 day of **July 2001**

ADAMS & ADAMS
APPLICANTS PATENT ATTORNEYS

The duplicate will be returned to the applicant's address for service as proof of lodging but is not valid unless endorsed with official stamp

OFFICIAL DATE STAMP
REGISTRAR OF PATENTS, DESIGNS, TRADE MARKS AND COPYRIGHT
2001 -07- 11
REGISTRATEUR VAN PATENTE, MODELLE, HANDELSMERKE EN OUTEURSREG
REGISTRAR OF PATENTS

A&A P201

A & A Ref No: V14729

ADAMS & ADAMS
PATENT ATTORNEYS
PRETORIA

FORM P6

REPUBLIC OF SOUTH AFRICA
Patents Act, 1978

PROVISIONAL SPECIFICATION

(Section 30 (1) - Regulation 27)

21	01	OFFICIAL APPLICATION NO

20015700

22	LODGING DATE

11 July 2001

71	FULL NAME(S) OF APPLICANT(S)

ROUSSOS, Deborah Kim

72	FULL NAME(S) OF INVENTOR(S)

ROUSSOS, Deborah Kim

54	TITLE OF INVENTION

"CELL ENUMERATION"

THIS INVENTION relates to the enumeration of CD4+ T cells in a cell sample, for example in a bone marrow cell sample, a body fluid sample, a disaggregated tissue sample, a tissue fine needle aspiration sample or, in particular, a blood sample. More particularly, the invention relates to a method suitable for, but not limited to, enumerating the number of CD4+ T cells in a blood sample.

According to the invention, there is provided a method of enumerating the number of CD4+ T cells in a cell sample, the method comprising the steps of:

counting all the white blood cells in the cell sample to obtain the white blood cell population thereof;

determining the proportion or percentage of CD4+ T cells in the white blood cell population in the sample; and

calculating the number of CD4+ T cells in the sample by relating the proportion or percentage of CD4+ T cells to the white blood cell population in the sample, thereby to establish the population of CD4+ T cells in the sample.

Counting the white blood cells (CD45 positive leucocytes) in the sample will naturally involve identifying the white blood cells in the sample, and any suitable known, conventional or established method may be used for such counting (and identification), using any suitable haematology analyser (or flow cytometer). For example, identification of nuclear acid (DNA) staining may be employed as a method of counting the white blood cell population (i.e. the white cell count) in the sample. The white blood cell population in the sample may be defined by flow cytometry, either immunophenotypically as CD45+ cells, or (using a technique substantially similar to DNA staining) as nucleated cells.

Actual calculating of the number of CD4+ T cells in the sample may be performed by multiplying the white blood cell population in the sample by a factor which is the proportion of CD4+ T cells in the white blood cell population when the proportion of CD4+ T cells of the white blood cell population is expressed as a fraction. If the proportion of CD4+ T cells in the white blood cell population is expressed as a percentage, then the white blood cell population will be multiplied by a factor which is a hundredth of the percentage (% x 10^{-2}).

The invention thus provides a dual-platform method of establishing the number of CD4+ T cells in the sample, using two separate parameters.

Counting the white blood cells in the sample may, as indicated above, be by means of a suitable haematology analyser. Any suitable haematology analyser may be employed, examples being those available under the trade names GenS™, CellDyn™ 4000, and XE2100™, manufactured respectively by Beckman Coulter, Inc. (GenS™), Abbott (CellDyn™ 4000) and Sysmex Corporation (XE2100™). In turn, determining the proportion of CD4+ T cells in the white blood cells in the sample may, as indicated above, be by means of a suitable flow cytometer. Any suitable flow cytometer may be employed, examples being those available under the trade names EPICS™ XL and FACSCalibur™, manufactured respectively by Beckman Coulter, Inc. (EPICS™ XL) and BD Biosciences (FACSCalibur™).

While any suitable technique for counting the white blood cells in the sample may be employed, counting said white blood cells may, for example, be by a size discrimination technique or by an impedance measuring technique.

Similarly, while any suitable technique for determining the proportion of

CD4+ T cells in the white blood cells in a sample may be employed, determining the proportion of CD4+ T cells in the white blood cells in the sample may, for example, be by means of flow-cytometric 90°/side scatter with CD4 expression. While the counting of the white blood cells and the determining of the proportion of CD4+ T cells in the sample may be carried out on separate instruments, namely a haematology analyser and a flow cytometer, it may be possible to combine the functions of such instruments so that both the white blood cell count and the determination of the proportion or percentage of CD4+ T cells of the white blood cells can conveniently be carried out on a single or common instrument.

In a particular embodiment of the invention a dual-platform (i.e. double- or twin-platform) method may be employed, involving two separate instruments to obtain the CD4+ T cell population (i.e. the absolute CD4+ T cell count) in the sample, namely a haematology analyser to obtain the white cell count and a flow cytometer to obtain the fraction or percentage of CD4+ T cells. The white cell count obtained from the haematology analyser is then multiplied by the CD4+ T cell fraction or percentage (as indicated above), measured as a fraction of the population of white blood cells (defined as the CD45+ (positive-expressing) cells) obtained from the flow cytometer. This calculation facilitates the generation of the absolute CD4+ T cell count.

Thus, for example, a Beckman Coulter GenS™ haematology analyser may be used to obtain a white cell count on a blood sample, followed by measurement of the CD4+ T cell fraction or percentage of the white blood cell population (defined by the CD45+ expression thereof) using a Beckman Coulter EPICS™ XL flow cytometer, on the same blood sample.

Instead, a single-platform method may be employed, involving a single

instrument, the total white cell count and the CD4+ T cell fraction or percentage of the total white cell population being obtained on a single instrument (which may be either a flow cytometer or a haematology analyser). This may be effected by counting the white blood cells directly on a haematology analyser having a facility for fluorescence measurement which can be altered to effect measurement of the fraction or proportion of CD4+ T cells of the total white cell population. Alternatively, the CD4+ T cell fraction or percentage of the total white cell population may be measured directly on a flow cytometer for a blood sample, which flow cytometer has had a suitable (commercially available) bead counting preparation (such as Beckman Coulter Flow-Count™ Fluorospheres) added thereto, the beads being counted simultaneously and being used as a standard from which the absolute number of CD4+ T cells can be calculated.

The invention will now be described, by way of non-limiting illustration, with reference to the following explanatory Examples, with reference to the accompanying drawings, Histograms (I) - (V).

EXAMPLE 1

DUAL-PLATFORM METHOD

A full blood sample was collected from a consenting adult in ethylene diamine tetra-acetic acid (EDTA). This sample was delivered to the laboratory within 6 hours of venesection. Upon receipt in the laboratory the sample was subjected to a white blood cell count performed on a Beckman Coulter GenS™ haematology analyser. A white cell count of 4.80 x $10^9/\ell$ (liter) was obtained.

Appropriate internal quality control as recommended by the supplier was performed daily on this instrument to ensure that the white cell count was accurate.

After the white blood cell count was obtained, blood taken from the same sample was prepared for flow cytometric analysis. Flow cytometric preparation involved dispensing a well - mixed 100 microlitre ($\mu\ell$) aliquot of whole blood into the bottom of a 12 x 75mm test tube. Anti-CD4 phycoerthryrin (PE) monoclonal antibodies and CD45 fluorescein - isothiocyanate (FITC) monoclonal antibodies were added (both obtained from Immunotech, Beckman Coulter, Inc., Miami, Florida) according to the supplier's recommendations and incubated for 10 minutes at room temperature, in the dark.

The tube containing the 100$\mu\ell$ of blood with the added CD45 and CD4 antibodies was then prepared for flow cytometric analysis on a Beckman Coulter Q-Prep / ImmunoPrep™ Reagent System and Workstation, as directed by the supplier. This preparative step included adding a red blood cell lysing agent, a stabiliser and a fixative. The sample was then analysed on a Beckman Coulter EPICS™ XL flow cytometer as set forth hereunder. Appropriate internal quality control including proper alignment and standardisation for light scatter and fluorescence intensity as well as colour compensation as recommended by the supplier was performed daily on this instrument to ensure that the flow cytometry results were accurate.

Thus total leucocytes were first identified on a [CD45 FITC *vs.* side scatter (SS)] display (Region A in Histogram (I)). All CD45 + gated events in Region A (the total number of leucocytes) were then displayed in Histogram (II), containing only the CD45 + cells, using a [CD4 PE *vs.* SS] display where CD4 + + low side scatter lymphoid cells, were identified (Region B in Histogram (II)). A CD4 + T cell percentage of 16.5% was noted.

The absolute CD4+ T cell count was obtained by multiplying the white cell count by the CD4+ T cell fraction obtained by flow cytometry i.e. 4.8 x $10^9/\ell$ multiplied by 16.5% divided by 100. This gave 0.792 x 10^9 CD4+ T cells/ℓ (or 792 CD4+ T cells/$\mu\ell$).

In this method, the total leucocytes served as a common denominator for dual-platform absolute CD4+ T cell counting instead of using the lymphoid population as the common denominator, which, is the state of the art.

The gating strategy employed also made possible the calculation of the CD4+ T cell percentage of total lymphocytes, i.e. the number of events in Gate B (Histogram (II)) divided by the number of events in Gate C. Thus, 752 CD4+ events noted in Gate B were divided by 1416 CD45+ bright events noted in Gate C (Histogram(I)), i.e. 752/1416 (0.531 or 53.1%). This CD4 cell percentage of total lymphocytes, irrelevant to the present invention, was nevertheless available in case it was clinically relevant, e.g. in paediatric cases.

EXAMPLE 2

SINGLE-PLATFORM METHOD

A full blood sample was collected in EDTA from a consenting adult patient. This sample was delivered to the laboratory within 6 hours of venesection. Upon arrival in the laboratory the sample was prepared for flow cytometric analysis. Flow cytometric preparation involved dispensing (reverse pipetting) a well- mixed 100 microlitre ($\mu\ell$) aliquot of the sample (whole blood) into the bottom of a 12 X 75mm test tube.

Anti-CD4 PE monoclonal antibodies and CD45 FITC monoclonal antibodies were added (both obtained from Immunotech, Beckman Coulter, Inc., Miami, Florida) according to the supplier's recommendations and incubated for 10 minutes at room temperature, in the dark. The tube containing the 100$\mu\ell$ of blood with the added CD45 and CD4 antibodies was then prepared for flow cytometric analysis using the above mentioned Q-Prep / ImmunoPrep™ Reagent System and Workstation, as directed by the supplier.

After this whole blood preparation step, which included adding a red cell lysing agent, a stabiliser and a fixative, commercially available bead reagents (Flow-Count™ Fluorospheres obtained from Beckman Coulter, Inc., Miami, Florida) were added to the sample as directed by the supplier. This Fluorosphere addition step involved a using very well mixed 100 $\mu\ell$ aliquot of the Flow-Check™ Fluorospheres. The assayed concentration of the Fluorospheres was stated to be 1000/$\mu\ell$. The Fluorospheres were added by a reverse pipetting technique with careful attention not to pipette air bubbles. Good reverse pipetting technique was crucial to the accuracy and precision of these test results. The sample was analysed within 2 hours of the addition of the Fluorospheres and was well mixed prior to flow-cytometric analysis.

The sample was then analysed on a Beckman Coulter EPICS™ XL flow cytometer until 1000 Fluorospheres were counted. Appropriate internal quality control including proper alignment and standardisation for light scatter and fluorescence intensity as well as colour compensation, as recommended by the supplier was performed daily on this instrument to ensure that flow cytometry results were accurate. In this analysis total leucocytes were first identified on a [CD45 FITC vs. side scatter (SS)] display (Region A in Histogram (III). All CD45 gated cells (i.e. gated events) in Region A (the total number of leucocytes) were then displayed in

Histogram (IV), containing only the CD45 + cells, using a [CD4 PE vs. SS] display where CD4 + +, low side scatter lymphoid cells were identified (Region B in Histogram (IV)). The Fluorosphere population was shown in a separate histogram of [Forward Scatter (FS) vs Fluorospheres] the latter of which were detected on log scale of FL4 (fluorescence detector number 4) (Region D, Histogram (V)).

The absolute CD45 + cell count was calculated as directed by the Fluorosphere manufacturer's instructions (Beckman Coulter Flow-Count™), .i.e. total number of cells counted divided by the total number of Fluorospheres counted, and then multiplied by the Flow-Count™ Fluorosphere assayed concentration, gave the absolute count/$\mu\ell$.

The CD45 + leucocyte count was therefore calculated as follows:
The Region A count of 4737 (total CD45 + cells counted) was divided by the Region D count of 1000 (Total Fluorospheres counted), and then multiplied by 1000/$\mu\ell$ (the Fluorosphere assayed concentration), which gave 4737/$\mu\ell$ or 4.737 x $10^9/\ell$) (the absolute CD45 + leucocyte or white cell count). The absolute CD4 + T cell count was obtained by multiplying this calculated CD45 + white cell count by the percentage of CD4 + +, low side-scatter T cells counted within the CD45 + leucocyte population (Region B, Histogram (IV)) i.e 4737/$\mu\ell$ multiplied by 16.2% (i.e. by 0.162). This gave 766 CD4 T cells/$\mu\ell$ (or 0.766 x 10^9 CD4 + T cells/ℓ).

In this method, the total CD45 + leucocyte count served as the base line for the absolute CD4 + T cell counting instead of using the lymphoid population or CD3 positive lymphocyte count as the base line, which is the state of the art.

The gating strategy employed also made possible the calculation of the CD4 + T cell percentage of total lymphocytes i.e. the number of cells in Gate B (Histogram

(IV)) divided by number of cells in Gate C (Histogram III)). Thus, 766 events noted in Gate B were divided by 1421 CD45+ bright events noted in Gate C, i.e. 766 divided by 1421 (0.539 or 53.9%). This value was not however used in the generation of the CD4+ count, and was merely extra information available in case the CD4 percentage of lymphocytes was clinically relevant, e.g. in paediatric cases.

Regarding Histograms (I) - (IV), if is to be noted that Histogram (I) is identical to Histogram (III)), and Histograms (II) and (IV) are identical to each other, with the exception of the presence of beads in Histogram (III).

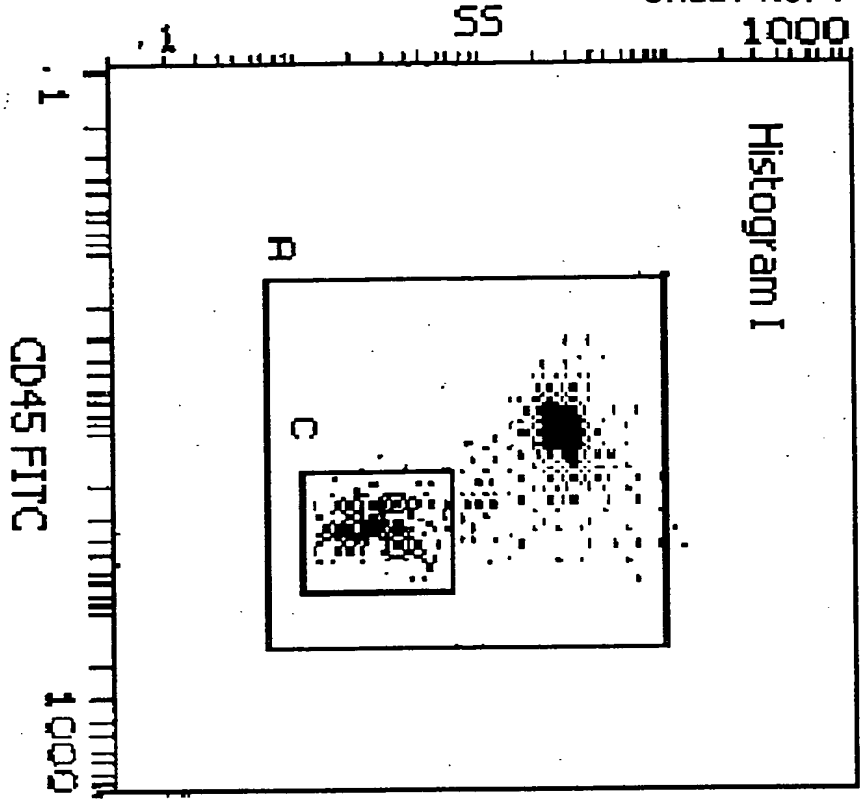
Advantages of the invention, particularly as described with reference to the above Example(s), are that the use of a count of the white blood cell population incorporating the use of CD45 expression as the basis for calculating the number of CD4+ T cells in cell samples facilitates accurate determinations after delays of up to several days after sample collection. Both CD45 and CD4+ T cell expressions are preserved with only minor losses of fluorescence intensity for up to 48 - 60 hours, even after loss of forward scattering properties. Indeed both the CD45+ leucocyte- and the side-scattering features of white blood cells promise to be retained for up to 5 days after sample collection. By virtue of including both the CD45+ leucocyte- and side- scattering parameters, technical errors can be avoided and irrelevant cellular events arising from, for example, monocytes or red blood cells, can be excluded while relevant lymphoid cells e.g. those with apoptotic scatter features, can be included. The approach of the present invention is particularly amenable to paediatric samples where small quantities of blood are available. In the absence of CD45+ leucocyte staining, unlysed and nucleated red blood cells may drastically interfere with the definition of both CD4+ T lymphocyte absolute counts and CD4+ T lymphocyte fraction- or percentage values. In infants and children the CD4+ T lymphocyte percentage counts are used as the clinically

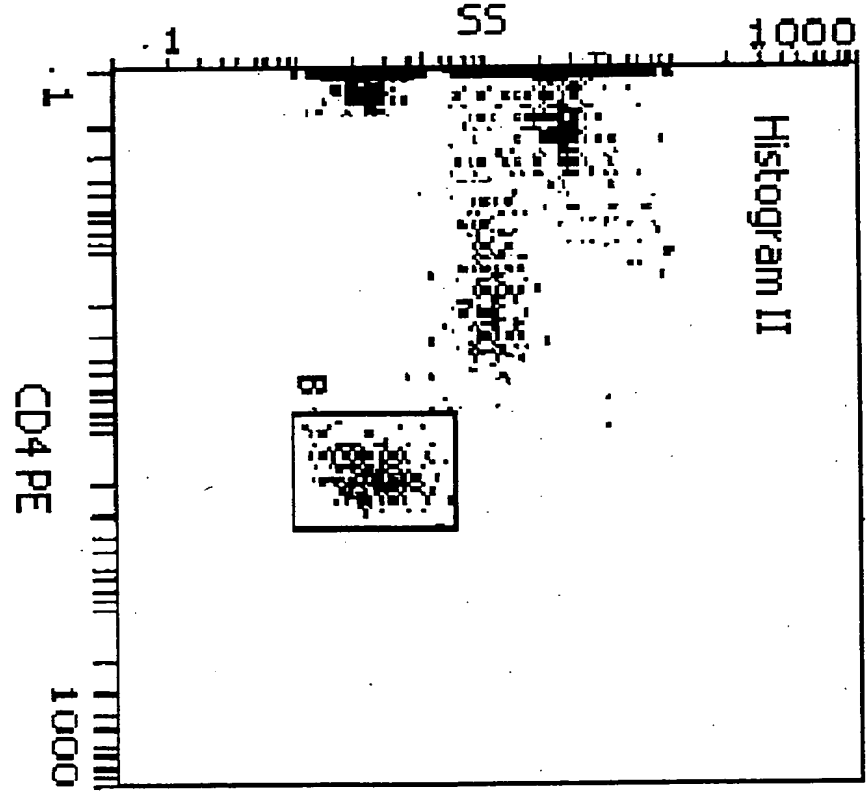
relevant parameter because of the age-dependent variability of the CD4+ T absolute counts. Use of CD45+ leucocyte counts as a basis facilitates use of precise CD4+ T lymphocyte fraction- or percentage values by identifying the CD4++ T cells as a function the bright CD45++ leucocyte cells.

The method of the present invention is relative robust, reproducible and accurate while being easy to use and comparatively inexpensive and easily implemented.

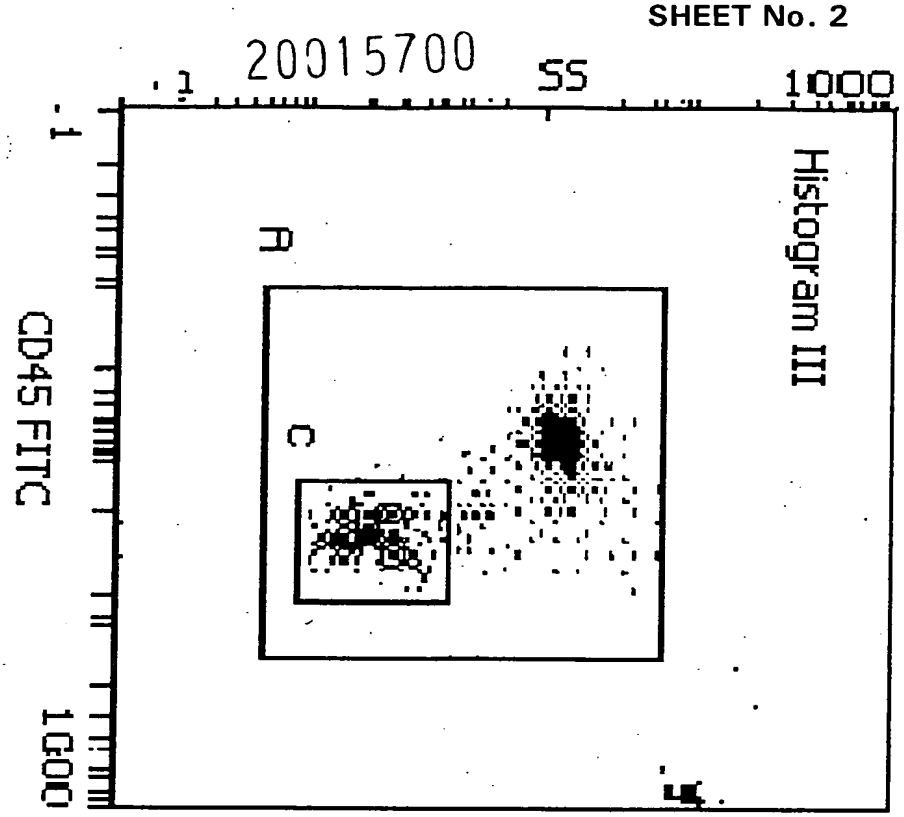
DATED THIS 11th DAY OF JULY 2001

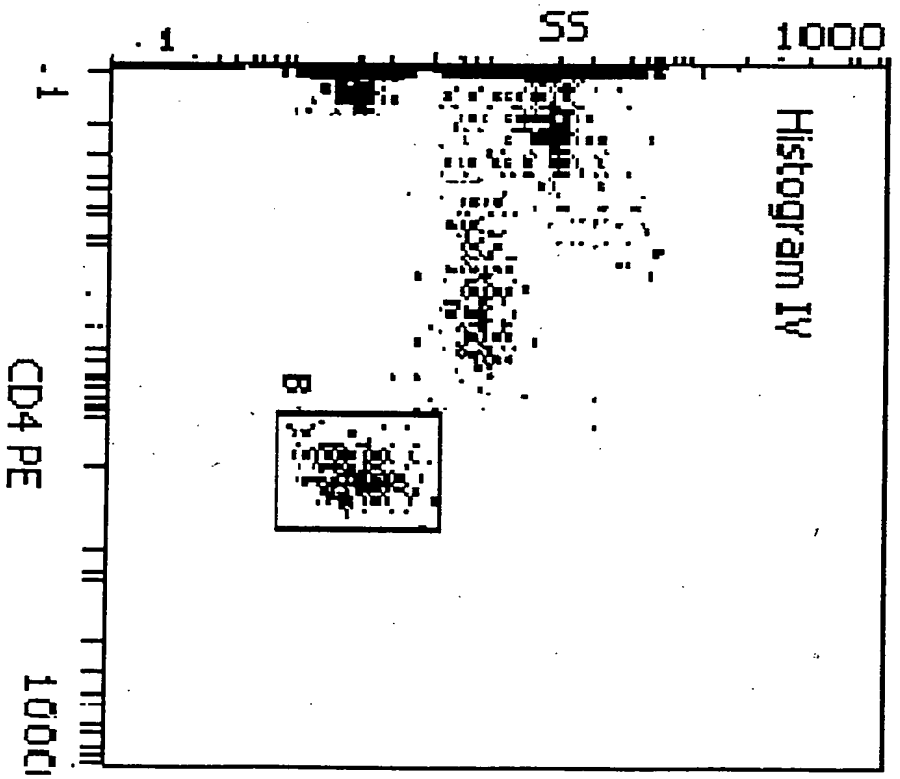
AV vR SCHWEIZER
ADAMS & ADAMS
APPLICANT'S PATENT ATTORNEYS





A SCHWEIZER
ADAMS & ADAMS
APPLICANT'S PATENT ATTORNEYS

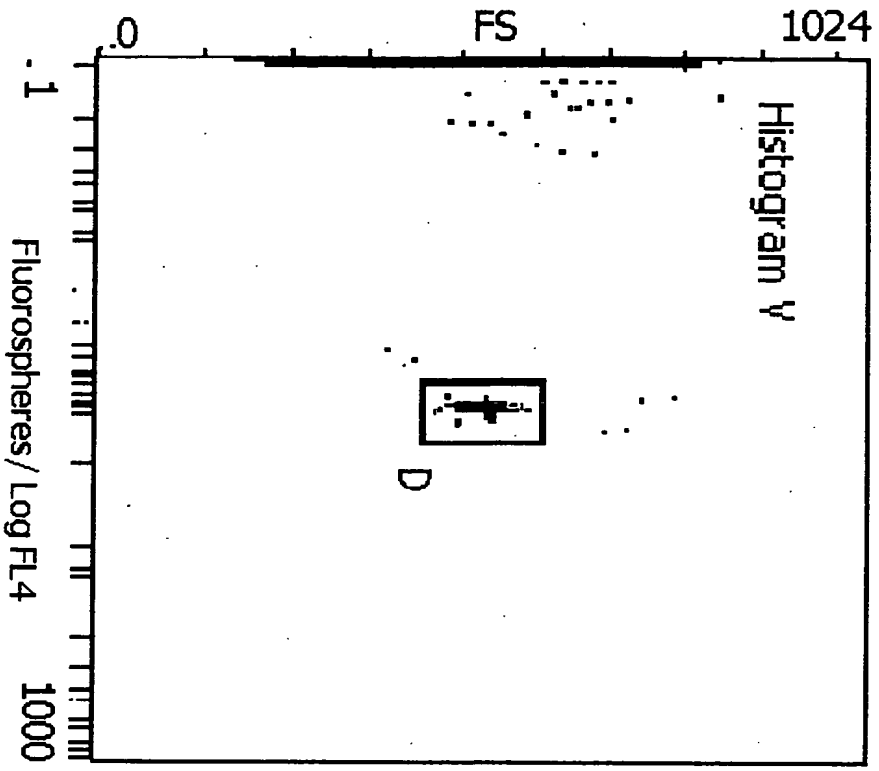




A SCHWEIZER
ADAMS & ADAMS
APPLICANT'S PATENT ATTORNEYS

ROUSSOS, Deborh Kim

THREE SHEETS
SHEET No. 3



A SCHWEIZER
ADAMS & ADAMS